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First report of vancomycin resistant Enterococcus faecalis and Enterococcus faecium isolated from water buffalo clotted cream in Turkey

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First report of vancomycin resistant *Enterococcus faecalis* and *Enterococcus faecium* isolated from water buffalo clotted cream in Turkey

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ABSTRACT: This study aimed to isolate the *Enterococcus* species from homemade water buffalo clotted cream sold in Afyonkarahisar, investigate the vancomycin resistance genes in isolated species by PCR, and determine the antibiotic resistance of isolates to some commonly used antibiotics in Turkey. A total of 107 buffalo clotted cream samples sold in public bazaars of Afyonkarahisar were collected. Following the phenotypic identification using a commercial identification kit, PCR was applied to isolates using species-specific primers. Forty *Enterococcus* isolates were obtained from 107 samples by PCR. Out of 40 isolates, 31 (77.5%) and 9 (22.5%) were identified to be *E. faecalis* and *E. faecium*, respectively. In the 40 isolates tested, one *vanA* (2.5%) and 14 *vanB* (35%) genes were identified. Among isolates harbouring the *vanB* gene, 10 and four were found to be *E. faecalis* and *E. faecium,* respectively. Thus, while the *vanB* gene positivity was found to be 32.3% in *E. faecalis* isolates and 44.4% in *E. faecium* isolates, the *vanA* gene positivity was determined to be 11.1% in *E. faecium* isolates. While all of isolates were phenotypically resistant to streptomycin and fusidic acid, high resistance rates were also determined to kanamycin (87.5%), cephalothin (80%), erythromycin (80%), gentamicin (77.5%), tetracycline (75%) and vancomycin (60%). Resistance to all tested antibiotics except teicoplanin was determined in *E. faecalis* isolates and except chloramphenicol in *E. faecium* isolates harbouring the vancomycin resistance genes. In conclusion, the buffalo clotted cream offered for consumption in Afyonkarahisar may be a potential risk for public health in terms of VRE species. To our knowledge, this is the first study showing the presence of VRE in clotted cream samples in Turkey.

Keywords: Buffalo clotted cream; *Enterococcus* spp.; PCR; vancomycin resistance

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INTRODUCTION

Enterococci found in soil, water, plants, gastrointestinal tracts of humans and warm-blooded animals, foods of animal origin, especially dairy products, are microorganisms that can be used as a starter or probiotic cultures in the food industry due to their metabolic and biotechnological properties (Giraffa, 2002; Fisher and Phillips, 2009; Krawczyk et al., 2021). On the other hand, enterococci are known to be important pathogens causing nosocomial bacteremia and community-acquired infections in clinical microbiology (Moellering, 1992; Mundy et al., 2000; Guzman Prieto et al., 2016). In addition, multiple antibiotic resistance is widespread in food-derived *Enterococcus* species, especially *Enterococcus faecalis* and *Enterococcus faecium* (Mundy et al., 2000; Shepard and Gilmore, 2002). After the first report of vancomycin-resistant *Enterococcus* (VRE) strains (Uttley et al., 1988), a major concern has developed since vancomycin is considered as the last alternative for the treatment of multiple resistant infections (Adams et al., 2016). It has been shown that VRE species are among the most important hospital pathogens that can also be isolated from animal populations in recent years, and that isolates carrying antibiotic resistance genes can be transferred to humans through animals and foods of animal origin (Nilsson, 2012; Ahmed and Baptiste, 2018; Wist et al., 2020). Research on the presence of Enterococci or VRE in dairy products of animal origin has mostly been conducted on traditional or nontraditional cheese samples, and *E. faecalis* and *E. faecium* have been reported to be the most commonly isolated species from dairy products (Giraffa et al., 2000; Jurkovic et al., 2006; Trivedi et al., 2011; Sanlibaba and Senturk, 2018).

Afyonkarahisar is a province of Turkey famous for a clotted cream made from water buffalo milk, originally known as "Afyon kaymak". This traditional Turkish dairy product has an important marketspace in terms of both the manufacture region and the country. In the production of traditional homemade buffalo clotted cream, the buffalo milk is first filtered after milking, and then the milk is taken into cream pans made of aluminum or tinned copper, and preheated up to 70-75 °C. Following the pre-heating, the milk in the pans is heated to 90-95 °C with continuous stirring for 4-5 hours. At the end of this period, the milk is poured into 8-10 cm deep pans from a certain height to provide the foamy and porous cream formation. The pans are cooled down to 40-45 ºC and briefly reheated to 70-75 °C. Afterwards, they are kept in a cold room for 24 hours, and the cream layer is well formed. Small pieces of ice are sprinkled on the surface of the pan. The cream is sliced into four pieces with a sharp knife, packaged and presented to the consumer. Generally, previous research on clotted cream has focused on its microbiological and chemical quality (Siriken and Erol, 2009; Şenel, 2011), but the presence of Enterococci or VRE in water buffalo clotted cream samples has not been investigated by molecular methods in Turkey. Therefore, the present study aimed to isolate the *Enterococcus* species from this sought-after Turkish dairy product, investigate the vancomycin resistance genes (*vanA, vanB*, *vanC1,* and *vanC2/C3*) in isolated species by PCR, and determine the antibiotic resistance of isolates to some antibiotics commonly used in Turkey.

MATERIALS AND METHODS

Phenotypic isolation and identification of *Enterococcus* spp. from buffalo clotted cream samples

In this study, a total of 107 homemade water buffalo clotted cream samples produced and sold in public bazaars of Afyonkarahisar were analyzed. Samples were collected under aseptic conditions and immediately transferred to the laboratory in a cool box. After homogenization of each sample, 10 g were taken from each sample and transferred into 90 mL of Enterococcosel broth (Bile Esculin Azide broth; Becton, Dickinson and Company, NJ, USA) containing 10% skimmed milk for pre-enrichment. The broths were then vortexed and aerobically incubated at 35 °C for 24 hours. After incubation the broths were vortexed again and 10 µL from each pre-enrichment broth were inoculated onto Enterococcosel agar (Bile Esculin Azide agar; Becton, Dickinson and Company, NJ, USA). The petri dishes were aerobically incubated at 35 °C for 24-48 hours. At the end of the incubation period, black pigmented colonies grown on agar were examined macroscopically and microscopically. For this purpose, colony morphology, gram staining, catalase activity, and growth ability in nutrient broth containing 6.5% NaCl of suspected colonies were evaluated (Quinn et al., 1999; Holt et al., 2000; Jokovic et al., 2008). The certain phenotypic identification of isolates was performed using the BBL Crystal™ Identification Systems Gram-Positive ID kit (Becton, Dickinson and Company, NJ, USA) as described by the manufacturer. The isolated isolates were stored at -20°C in trypticase soy broth containing 15% glycerol to be used for DNA extraction.

Genotypic identification of* Enterococcus spp. and determination of vancomycin resistance genes (vanA, vanB, vanC1, vanC2/C3) *by PCR

DNA extraction from all isolates was performed using GeneJET genomic DNA purification kit (Thermo Scientific, Lithuania) as described by the manufacturer. While a duplex PCR protocol was applied using species-specific primers for the identification of *E. faecalis* and *E. faecium* (Jackson et al., 2004), multiplex PCR included *vanA* (Dutka-Malen et al., 1995), *vanB* (Elsayed et al., 2001), *vanC1* (Dutka-Malen et al., 1995) and *vanC2/C3* (Satake et al., 1997) genes specific primers to detect any vancomycin resistance in the isolates. The oligonucleotide sequences and reference protocols used in this study are shown in Table 1.

A PCR mixture of 25 µL consisting 10x PCR buffer, 3 mM $MgCl_2$, 200 µM dNTP mix (Ampliqon A/S, Denmark), 1 mM of each primer (Eurofins Genomics, Ebersberg, Germany), 2 U Taq DNA polymerase (Ampliqon A/S, Denmark), 5 µL DNA template and deionized water was used. The amplification conditions used for the specific detection of *E. faecalis*, *E. faecium* and vancomycin resistance genes are shown

in Table 2. All amplification products were stained with ethidium bromide (5µL/mL) (Thermo Fisher Scientific, USA) and following 1.5% agarose gel electrophoresis at 110 V for 70 minutes were visualized in a UV-transilluminator.

Antibiotic susceptibility testing

The antibiotic resistance of all identified isolates to 14 antimicrobial agents was tested on Mueller Hinton agar (MHA; Oxoid Limited, Hampshire, UK) using Kirby-Bauer disc diffusion method according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2022). From pure culture, selected colonies of bacteria were taken and transferred to a tube containing sterile tryptone soya broth (Oxoid Limited, Hampshire, UK) and incubated at 37 °C until the turbidity of the suspension became adjusted to a McFarland standard 0.5. Within 15 minutes after adjusting the turbidity of the inoculum suspension, a sterile cotton swab was dipped into the adjusted suspension. The swab was rotated several times and pressed firmly on the inside wall of the tube above the fluid level. The suspension was inoculated onto the dried surface of MHA plates by streaking the swab

Table 1. Oligonucleotide sequences used in this study

Target gene		Oligonucleotide sequence (5'−3')	Product size (bp)	Reference
E. faecalis	Forward	ACTTATGTGACTAACTTAACC	360	Jackson et al., 2004
	Reverse	TAATGGTGAATCTTGGTTTGG		
E. faecium	Forward	GAAAAAACAATAGAAGAATTAT	215	Jackson et al., 2004
	Reverse	TGCTTTTTTGAATTCTTCTTTA		
vanA	Forward	GGGAAAACGACAATTGC	732	Dutka-Malen et al., 1995
	Reverse	GTACAATGCGGCCGTTA		
vanB	Forward	AAGCTATGCAAGAAGCCATG	536	Elsayed et al., 2001
	Reverse	CCGACAATCAAATCATCCTG		
vanC1	Forward	GGTATCAAGGAAACCTC	822	Dutka-Malen et al., 1995
	Reverse	CTTCCGCCATCATAGCT		
vanC2/C3	Forward	CGGGGAAGATGGCAGTAT	484	Satake et al., 1997
	Reverse	CGCAGGGACGGTGATTTT		

Table 2 Amplification conditions used in this study

Step	Cycle		Temperature		Time	
	E. faecalis, E. faecium	*vanA, vanB, vanC1, vanC2/C3*	*E. faecalis, E. faecium*	*vanA, vanB, vanC1, vanC2/C3*	*E. faecalis, E. faecium*	*vanA, vanB, vanC1, vanC2/C3*
Initial denaturation	1	1	95 °C	94 °C	4 min	2 min
Denaturation	35	30	95 °C	94 °C	30 sec	60 sec
Annealing	35	30	55 °C	54 °C	60 sec	60 sec
Extension	35	30	72 °C	72 °C	60 sec	60 sec
Final extension	1	1	72 °C	72 °C	7 min	10 min

over the entire sterile agar surface. This procedure was repeated by streaking two more times, rotating the plate approximately 60° each time to ensure an even distribution of inoculum. The antimicrobial discs were placed onto the surface of the inoculated agar plate using forceps. The plates were inverted and incubated at 37 °C for 18 hours for vancomycin and 24 hours for other antimicrobial discs within 15 minutes after the discs were applied. Following incubation, plates were held a few inches above a black background illuminated with reflected light, except for vancomycin, which was read with transmitted light (plates were held up to light source). The inhibition zone diameters were measured three times using a ruler. The presence of a haze or any growth within the zone of inhibition was evaluated as positive for vancomycin resistance (CLSI, 2022). Tested antibiotics were amoxicillin/clavulanic acid (30μg), tetracycline (30μg), penicillin G (10U), erythromycin (15μg), cephalothin (30μg), gentamicin (10μg), vancomycin (30μg), ampicillin (10μg), chloramphenicol (30μg), streptomycin (10μg), ciprofloxacin (5μg), kanamycin (30μg), teicoplanin (30μg) and fusidic acid (10μg) (Oxoid Limited, Hampshire, UK). *E. faecalis* ATCC 51299 and *E. faecalis* ATCC 29212 were used as reference strains.

RESULTS

Phenotypic isolation and identification findings

In the present study, 61 bacterial isolates suspected in terms of *Enterococcus* were obtained from 107 water buffalo clotted cream samples. However, 40 out of the 61 isolates were determined to be *Enterococcus* by a commercial identification kit.
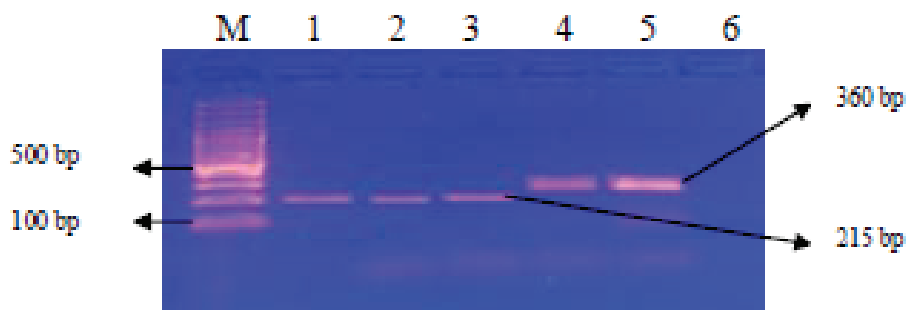
PCR findings

In duplex PCR used for the confirmation of phenotypically identified isolates, 31 and 9 of the isolates were typed to be *E. faecalis* and *E. faecium*, respectively. Thus, while *Enterococcus* isolation rate was 37.4% in 107 buffalo clotted cream samples, *E. faecalis* was isolated from 29% and *E. faecium* from 8.4% of the samples. The gel electrophoresis image for *E. faecalis* (360 bp) and *E. faecium* (215 bp) is shown in Figure 1.

According to multiplex PCR results, one *vanA* (2.5%) and 14 *vanB* (35%) genes were detected among the tested 40 isolates. Ten *E. faecalis* and four *E. faecium* isolates were positive for *vanB* gene, and one *E. faecium* isolate harboured the *vanA* gene. The *vanB* gene positivity was found to be 32.3% in *E. faecalis* isolates and 44.4% in *E. faecium* isolates, and the *vanA* gene positivity was determined to be 11.1% in *E. faecium* isolates. The *vanC1* and *vanC2/C3* genes were not found in any of the isolates (Table 3). The PCR products for *vanA* (732 bp) and *vanB* (536 bp) genes are shown in Figure 2.

Antibiotic susceptibility testing findings

All of 40 isolates typed by PCR were resistant to streptomycin and fusidic acid. High resistance rates were also determined towards kanamycin (87.5%), cephalothin (80%), erythromycin (80%), gentamicin (77.5%), tetracycline (75%) and vancomycin (60%). The antibiotic resistance rates in *E. faecalis* and *E. faecium* isolates are shown in Table 4.

The presence of vancomycin resistance genes determined by PCR was inconsistent with the phenotypic resistance rate obtained by the Kirby-Bauer disc
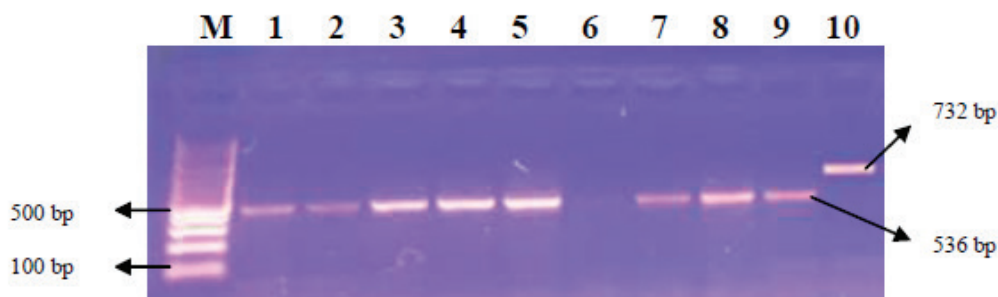

Figure 1 PCR findings of *E. faecalis* and *E. faecium* test isolates. M: 100 bp DNA ladder; Lanes 1-3: *E. faecium* (215 bp); Lanes 4,5: *E. faecalis* (360 bp); Lane 6: sterile distilled water

diffusion test. Although 24 of 40 isolates were phenotypically resistant to vancomycin, only 15 (37.5%) of 40 isolates harboured the resistance genes. Also, *E. faecalis* isolates were found to be resistant to all tested antibiotics except teicoplanin and *E. faecium* isolates with vancomycin resistance genes were resistant to all tested antibiotics except chloramphenicol (Table 4).

Figure 2 PCR findings of *vanA* and *vanB* genes in *E. faecium* and *E. faecalis* test isolates. M: 100 bp DNA ladder; Lanes 1-5: *vanB* gene positive *E. faecalis* isolates (536 bp); Lanes 6-9: *vanB* gene positive *E. faecium* isolates (536 bp); Lane 10: *vanA* gene positive *E. faecium* isolate (732 bp)

Table 3 Distribution of vancomycin resistance genes

Species (n)	vanA n (%)	vanB n (%)	vanC1 n (%)	vanC2/C3 n (%)
E. faecalis (31)	0	10 (32.3)	0	0
E. faecium (9)	1 (11.1)	4 (44.4)	0	0
Total (40)	1 (2.5)	14 (35.0)	0	0

Table 4 Antibiotic resistance of Enterococci and VRE isolates

Antibiotic	E. faecalis (n=31)			E. faecium (n=9)			Total (n=40)			VR E. faecalis (n=10) (vanB)			VR E. faecium (n=5) (1 vanA, 4 vanB)			Total VRE (n=15)		
	R n (%)	I n (%)	S n (%)	R n (%)	I n (%)	S n (%)	R n (%)	I n (%)	S n (%)	R n (%)	I n (%)	S n (%)	R n (%)	I n (%)	S n (%)	R n (%)	I n (%)	S n (%)
Amoxicillin/clavulanic acid (30µg)	4 (12.9)	2 (6.5)	25 (80.6)	4 (44.4)	0	5 (55.6)	8 (20)	2 (5)	30 (75)	1 (10)	0	9 (90)	1[#] (20)	0	4 (80)	2 (13.3)	0	13 (86.7)
Tetracycline (30µg)	24 (77.4)	1 (3.2)	6 (19.4)	6 (66.7)	3 (33.3)	0	30 (75)	4 (10)	6 (15)	9 (90)	1 (10)	0	4[#] (80)	1 (20)	0	13 (86.7)	2 (13.3)	0
Penicillin G (10U)	12 (38.7)	*-	19 (61.3)	4 (44.4)	*-	5 (55.6)	16 (40)	*-	24 (60)	5 (50)	*-	5 (50)	2[#] (40)	*-	3 (60)	7 (46.7)	*-	8 (53.3)
Erythromycin (15µg)	26 (83.9)	3 (9.6)	2 (6.5)	6 (66.7)	2 (22.2)	1 (11.1)	32 (80)	5 (12.5)	3 (7.5)	9 (90)	1 (10)	0	4[#] (80)	1 (20)	0	13 (86.7)	2 (13.3)	0
Cephalothin (30µg)	25 (80.6)	4 (12.9)	2 (6.5)	7 (77.8)	2 (22.2)	0	32 (80)	6 (15)	2 (5)	9 (90)	1 (10)	0	4[#] (80)	1 (20)	0	13 (86.7)	2 (13.3)	0
[a]Gentamicin (10µg)	24 (77.4)	5 (16.1)	2 (6.5)	7 (77.8)	1 (11.1)	1 (11.1)	31 (77.5)	6 (15)	3 (7.5)	9 (90)	1 (10)	0	4[#] (80)	1 (20)	0	13 (86.7)	2 (13.3)	0
Vancomycin (30µg)	16 (51.6)	10 (32.3)	5 (16.1)	8 (88.9)	1 (11.1)	0	24 (60)	11 (27.5)	5 (12.5)	10 (100)	0	0	4[#] (80)	1 (20)	0	14 (93.3)	1 (6.7)	0
Ampicillin (10µg)	17 (54.8)	*-	14 (45.2)	2 (22.2)	*-	7 (77.8)	19 (47.5)	*-	21 (52.5)	4 (40)	*-	6 (60)	1[#] (20)	*-	4 (80)	5 (33.3)	*-	10 (66.7)
Chloramphenicol (30µg)	12 (38.7)	7 (22.6)	12 (38.7)	2 (22.2)	2 (22.2)	5 (55.6)	14 (35)	9 (22.5)	17 (42.5)	1 (10)	1 (10)	8 (80	0	1 (20)	4 (80)	1 (6.7)	2 (13.3)	12 (80)
Streptomycin (10µg)	31 (100)	*-	0	9 (100)	*-	0	40 (100)	*-	0	10 (100)	*-	0	5 (100)	*-	0	15 (100)	*-	0
Ciprofloxacin (5µg)	11 (35.5)	12 (38.7)	8 (25.8)	3 (33.3)	6 (66.7)	0	14 (35)	18 (45)	8 (20)	1 (10)	2 (20)	7 (70)	1[#] (20)	4 (80)	0	2 (13.3)	6 (40)	7 (46.7)
Kanamycin (30µg)	29 (93.5)	0	2 (6.5)	6 (66.7)	3 (33.3)	0	35 (87.5)	3 (7.5)	2 (5)	10 (100)	0	0	5 (100)	0	0	15 (100)	0	0
Teicoplanin (30µg)	7 (22.6)	13 (41.9)	11 (35.5)	5 (55.6)	2 (22.2)	2 (22.2)	12 (30)	15 (37.5)	13 (32.5)	0	0	10 (100)	1[#] (20)	0	4 (80)	1 (6.7)	0	14 (93.3)
Fusidic acid (10µg)	31 (100)	0	0	9 (100)	0	0	40 (100)	0	0	10 (100)	0	0	5 (100)	0	0	15 (100)	0	0

[a]Low-level gentamicin resistance; *-: No standard defined for zone diameter; [#]: *vanA* gene positive one isolate; R: Resistant; I: Intermediate S: Susceptible

DISCUSSION

The present study aimed to investigate the presence of vancomycin-resistant *Enterococcus* species in the water buffalo clotted cream, a traditional Turkish dairy product, and determine the antibiotic resistance of *Enterococcus* isolates to some commonly used antibiotics in Turkey.

The resistance of Enterococci to pasteurization temperatures and their ability to adapt to different conditions (low and high temperatures, high pH, and salt) allow these species to be found in foods produced from raw materials (meat or milk) and in heat-treated foods. Although fermented foods containing Enterococci were previously considered safe, the presence of these bacteria in foods has also been a major concern for the food industry and consumers in terms of food spoilage, food poisoning and hospital infections (Franz et al., 2011; McAuley et al., 2012; Câmara et al., 2020; Mariam, 2021). Studies investigating the presence of Enterococci in dairy products of animal origin have mostly been conducted on traditional or nontraditional cheese samples, and *E. faecalis* and *E. faecium* have been reported to be the most commonly isolated species from dairy products (Giraffa et al., 2000; Jurkovic et al., 2006; Trivedi et al., 2011; Sanlibaba and Senturk, 2018). However, there is limited research related to the isolation of Enterococci from clotted creams (Jokovic et al., 2008; Siriken and Erol, 2009). In a study from different regions of Serbia, it was reported that 102 of 374 bacterial isolates obtained from six homemade clotted cream samples originating from cow's milk were evaluated as suspected in terms of Enterococci presence, but after PCR analysis, 43 and 10 of the isolates were determined as *E. faecium* and *E. faecalis*, respectively (Jokovic et al., 2008). Siriken and Erol (2009) from Turkey found an *Enterococcus* isolation rate of 20% from 30 buffalo clotted cream samples analyzed by standard conventional methods. However, there was no study investigating the presence of *Enterococcus* species in buffalo clotted cream, a traditional dairy product, by molecular methods in Turkey. In the present study, 40 *Enterococcus* isolates were detected in 107 water buffalo clotted cream samples, and 31 (77.5%) and 9 (22.5%) of these species were determined as *E. faecalis* and *E. faecium*, respectively, by PCR. Although the isolates obtained in the presented study were consistent with previously reported isolates isolated from other dairy products and clotted cream samples, the isolation rates are different. The reason for this difference may be related to the sample type, number of samples, differences in isolation procedures, and regional differences in the origin of the isolates.

The most important problem in the treatment of infections caused by Enterococci is the development of resistance to vancomycin, which is the only treatment option in infections caused by strains with multiple antibiotic resistance. These bacteria are capable of transferring their resistance genes both among themselves and to other Gram-positive bacteria via conjugative plasmids and transposons, showing a wide host spectrum (Mundy et al., 2000; Partridge et al., 2018; Růžičkova et al., 2020). Transmission of resistance genes to humans after consumption of foods contaminated with VRE isolates carrying antibiotic resistance genes has caused the food chain to be accepted as a potential reservoir for transmission of VRE isolates from animals to humans (Giraffa, 2002; Heuer et al., 2006; Hammerum et al., 2010; Lawpidet et al., 2021). Most studies on VRE prevalence in dairy products have been conducted on various cheese samples. In their study, Giraffa et al. (2000) investigated the presence of VRE in 10 different Italian cheeses, and the phenotypic vancomycin resistance was reported to be 25% (n=26 VRE) in 102 *Enterococcus* isolates isolated from 22 cheese samples. In the same study, *vanA* positivity was determined in all 23 VR *E. faecium* and three VR *E. faecalis* isolates by PCR. Jurkovic et al. (2006) investigated the presence of *vanA* and *vanB* resistance genes in 177 *E. faecium* and 41 *E. faecalis* isolates isolated from bryndza cheeses native to Slovakia, but no genes were found in any of the isolates. Similarly, in another study, it was reported that phenotypic resistance and vancomycin resistance genes were not detected in 56 *E. faecalis* and 30 *E. faecium* isolates isolated from dairy products (Trivedi et al., 2011). In a study from Turkey, it was emphasized that high levels of phenotypic resistance to vancomycin were detected in *E. faecium* and *E. faecalis* isolates isolated from cheese samples, but none of the isolates carried the *vanA*, *vanB*, and *vanC1* genes (Elmalı and Can, 2018). In our study, 15 (37.5%) of 40 *Enterococcus* isolates isolated from 107 buffalo clotted cream samples were found to be VRE, of which one and 14 harboured the *vanA* and *vanB* genes, respectively. Ten of the isolates carrying the *vanB* gene were identified as *E. faecalis* and 4 as *E. faecium*, and one *E. faecium* isolate harboured the *vanA* gene. The high isolation rates of vancomycin resistance genes in *E. faecalis* (10/31; 32.2% for *vanB* gene) and *E. faecium* (4/9; 44.4% for *vanB* gene and 1/9; 11.1% for *vanA* gene) isolates was a remarkable finding. This result may be

related to the differences in sample type, number of samples, number of isolates isolated, primers and amplification conditions used in this study. Especially in researches conducted on clinical isolates, it was emphasized that *vanA* and *vanB*-type resistances are acquired and transferable, and the *vanA* resistance profile is more common in *E. faecium* isolates, while the *vanB* resistance gene is frequently harboured by *E. faecalis* isolates (Mathur and Singh, 2005; Courvalin, 2006; Braïek and Smaoui, 2019). The higher isolation of the *vanB* resistance gene compared to the *vanA* gene may be related to the higher number of *E. faecalis* isolates isolated in our study. In the present study, the *vanC1* and *vanC2/C3* genes were not determined in any of the tested isolates. Some researchers emphasize that *vanC*-type resistance encoded by *vanC1* and *vanC2/C3* genes is commonly known as the intrinsic characteristic of *Enterococcus gallinarum*, *Enterococcus casseliflavus*, and *Enterococcus flavescens* strains (Courvalin, 2006; Braïek and Smaoui, 2019). The fact that *E. gallinarum*, *E. casseliflavus,* and *E. flavescens* were not isolated from the clotted creams sampled in our study may explain the absence of *vanC1* and *vanC2/C3* genes.

Different resistance rates have been reported in various studies as regards *Enterococcus* isolates isolated from dairy products of animal origin, especially cheese. In many of these studies, the most common phenotypic resistance profiles of the isolates were against kanamycin (Sanlibaba and Senturk, 2018), gentamicin (Kročko et al., 2011), ampicillin (Kročko et al., 2011; Elmalı and Can, 2018; Sanlibaba and Senturk, 2018), tetracycline (Çıtak et al., 2004; Sanlibaba and Senturk, 2018), erythromycin (Çıtak et al., 2004; Kročko et al., 2011; Elmalı and Can, 2018), streptomycin (Çıtak et al., 2004) and ciprofloxacin (Sanlibaba and Senturk, 2018). However, there are few studies reporting high resistance to vancomycin (Çıtak et al., 2004; Elmalı and Can, 2018). In our study, according to the results of the antibiotic susceptibility testing performed using the Kirby-Bauer disk diffusion test, all isolates were resistant to streptomycin and fusidic acid. High resistance rates against kanamycin (87.5%), cephalothin (80%), erythromycin (80%), gentamicin (77.5%), tetracycline (75%), and vancomycin (60%) were also determined in the isolates (Table 4). It is known that while Enterococci show intrinsic resistance to cephalosporins, many β-lactam group antibiotics and aminoglycosides (low level), they can exhibit acquired type resistance to chloramphenicol, erythromycin, tetracycline and gly-

copeptide antibiotics (Çıtak et al., 2004; Mathur and Singh, 2005; Braïek and Smaoui, 2019). The 100% resistance rate obtained against streptomycin and the high resistance rate determined against gentamicin in our study are consistent with this fact. However, Lopes et al. (2003) that compared gentamicin resistance in animal isolates, dairy products isolates of animal origin and clinical isolates, reported that gentamicin resistance in dairy products may not be intrinsic but possibly the gentamicin resistance gene is transferred from clinical or commensal bacteria to these isolates. The same authors also emphasized that this may be a problem for isolates isolated from dairy products in the future. In our study, all of the isolates were also resistant to fusidic acid. The CLSI (2022) recommends that this antibiotic should not be used *in vivo* in enterococcal infections, even if isolates susceptible to fusidic acid have been observed *in vitro*. The phenotypic resistance rates obtained in our study differed from the rates reported by other studies. This result may be related to the sample type, the number of isolated isolates, the origin of the isolates, and regional differences in the preferred antibiotics in the veterinary field.

In this study, the phenotypic resistance rates obtained by the disk diffusion test against vancomycin and the presence of resistance genes obtained as a result of PCR were not compatible. Although 24 of 40 isolates were phenotypically resistant to vancomycin, only 15 (37.5%) of the isolates were determined to be VRE. Of the 31 *E. faecalis* isolates, 16 were found to be phenotypically resistant to vancomycin, but 10 isolates harboured the resistance gene. Similarly, while eight of nine *E. faecium* isolates were phenotypically resistant to vancomycin, only five (55.5%) isolates had resistance genes. Some authors (Sreeja et al., 2012; Madoshi et al., 2018) emphasized that the disc diffusion test may not be sufficient and reliable in determining resistance to vancomycin, similar to that recommended by the CLSI. In addition, other resistance genes or other mechanisms responsible for vancomycin resistance may have been effective in this discordance between phenotypic resistance and the presence of resistance genes (Mirzaei et al., 2013). In the present study, phenotypic resistance to all tested antibiotics except teicoplanin and chloramphenicol was determined in *E. faecalis* and *E. faecium* isolates harbouring the vancomycin resistance genes, respectively. One *E. faecium* isolate carrying the *vanA* gene showed phenotypic resistance to teicoplanin. However, such resistance was not determined in any of the

vanB gene positive isolates (Table 4). Some researchers emphasize that while *vanA*-type resistance represents a high level of resistance to vancomycin and teicoplanin, *vanB*-type resistance represents varying levels of resistance to vancomycin, but not to teicoplanin (Courvalin, 2006; Levine, 2006; Braïek and Smaoui, 2019). The low teicoplanin resistance observed in our study is consistent with this view.

CONCLUSION

The present study showed the presence of *vanA* and *vanB* resistance genes in *E. faecalis* and *E. faecium* isolates isolated from water buffalo clotted cream for the first time in Turkey using PCR. The *vanA* gene-positive *E. faecium* isolates are known to be more important, especially in nosocomial VRE infections. However, considering that both *vanA* and *vanB* genes are transferable and this can be mediated by foods of animal origin, buffalo clotted cream samples positive for VRE species may pose a potential risk to public health. In addition, multiple antibiotic resistance to the tested antibiotics was determined in VR *E. faecalis* and VR *E. faecium* isolates obtained from clotted cream samples. In order to prevent the dangerous rise of antibiotic resistance, which is a global problem, appropriate and specific antibiotic use should be encouraged after antibiotic susceptibility testing, and the resistance should be monitored regularly. Hopefully, the results of this study will contribute to future studies on the subject.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

ETHICS STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors.

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